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A Metamorphic Inducer in the Opisthobranch *Haminaea callidegenita:* Partial Purification and Biological Activity

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Abstract. Larvae of *Haminaea callidegenita* (Mollusca: Cephalaspidea) were induced to metamorphose by a compound found in the gelatinous matrix composing most of the egg mass. A functionally similar compound isolated from adult tissue also induced metamorphosis in *H. callidegenita* larvae. Opisthobranchs are frequently induced to metamorphose by a specific prey item or a substrate characteristic of the adult habitat, but this is the first known instance of metamorphosis occurring in response to a compound produced by adult conspecifics. The inducer was purified from egg mass jelly (EMJ) by high pressure liquid chromatography (HPLC) and was found to be smaller than 1000 Da, polar, non-proteinaceous, and very stable. We isolated a compound of identical activity from egg masses produced by four other opisthobranch species, suggesting that the same or chemically similar compounds are intrinsic to opisthobranch egg masses. However, only *H. callidegenita* larvae metamorphosed in response to EMJ. Competent larvae of five other mollusc species did not respond to the partially purified EMJ inducer but did respond to a specific substrate associated with each species. The presence of the inducer within the egg mass causes an unusual developmental pattern in *H. callidegenita,* a poecilogonous species that produces both swimming veliger and crawling juvenile offspring.

Introduction

Larvae of most benthic marine invertebrate species are induced to metamorphose by a cue, either chemical or physical, that identifies a specific habitat as suitable for juvenile or adult existence (reviewed by Pawlik, 1992). This cue, or metamorphic inducer, triggers a radical transformation of the morphology, physiology, ecology, and behavior of the larva to the juvenile stage. Chemical inducers are water-borne or tactile, and originate from conspecifics, prey species, or a characteristic micro- or macrobiotic substrate (bacterial film or an algal, invertebrate, or vertebrate species; see Pawlik, 1992). For example, most progress in the isolation of metamorphic inducers in opisthobranchs has involved two nudibranchs, both of which are induced to metamorphose by a highly specific adult prey species. Larvae of *Phestilla sibogae* metamorphose in response to a small, water-soluble molecule derived from the coral *Porites* (Hadfield, 1977; Hadfield and Scheuer, 1985), and those of *Eubranchus doriae* respond to a small, polar compound with glucosidic residues originating from the hydroid *Kirchenpaueria* (Bahamondes-Rojas, 1988; Bahamondes-Rojas and Dherbomez, 1990). Despite many attempts to purify metamorphic inducers in marine invertebrates in general, such inducers have been identified for only a few species and include a variety of chemical groups (see review by Pawlik, 1992; also Kitamura *et al.*, 1993).

The opisthobranch *Haminaea callidegenita* (*Haminaea* = *Haminoea* [*cf.* Giannuzzi-Savelli and Gentry, 1990]) is induced to metamorphose by a compound found in the gelatinous layers composing most of the egg mass (Gibson and Chia, 1989a). An unusual life history results in which half the siblings per egg mass metamorphose in response to egg mass jelly (EMJ; the metamorphic inducer) to hatch as crawling juveniles. The other half hatch as swimming veligers and, during a two-week planktonic period, gradually become competent to metamorphose in response to EMJ as well as to other substrates indicative of a juvenile

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food source (Gibson, 1993). Only EMJ induces intracapsular metamorphosis; veligers do not become sensitive to the other substrates until after hatching (Gibson and Chia, 1989a). The ecological consequence of this developmental pattern is that each parent simultaneously produces both dispersive (lecithotrophic veliger) and nondispersive (juvenile) offspring. Hatchling type depends on the presence of EMJ before hatching and the time at which metamorphic competence occurs (before or after hatching).

In this paper, we characterize the metamorphic inducer found in EMJ and describe purification techniques. The inducer was smaller than 1000 Da in molecular weight, polar, and stable to temperature and acid. We also describe the ecological distribution of the EMJ inducer that influences its activity. A functionally similar compound was extracted from adult *H. callidegenita* tissue as well as from the egg masses of other opisthobranch species. Metamorphic inducing activity of EMJ was specific to *H. callidegenita* larvae. We have focused on intracapsular metamorphosis only, because this is the basis for poecilogony in this species; substrates that induce metamorphosis after hatching are described elsewhere (Gibson, 1993).

Materials and Methods

Culture and bioassay procedures

Haminaea callidegenita were collected at Padilla Bay or Spencer Spit and maintained after collection at the University of Washington, Friday Harbor Laboratories, all in Washington State. Egg masses used in these assays were collected either in the field or within 24 h of natural spawning by animals maintained in the laboratory. Culture techniques for adults are described elsewhere (Gibson, 1993). Gastrulae were separated from the sausage-shaped egg mass by removing the jelly layers composing most of the mass because this jelly is known to induce metamorphosis within the egg mass (Gibson and Chia, 1989a). EMJ was removed from developing embryos with fine forceps, then the embryos were rinsed in 1-μm filtered seawater (fSW). Embryos were still contained by an embryonic capsule. Unlike the egg masses of other opisthobranchs, *H. callidegenita* egg masses fall apart easily and all traces of EMJ can be removed. Embryos were cultured in 100 ml of fSW at 17°C, and cultures were cleaned twice weekly. Hatching, defined as the escape of larvae or juveniles from the capsule, began about 14 days after spawning. In intact egg masses, hatchlings must also crawl through the jelly matrix; thus hatching takes slightly longer in intact masses (4–5 days; Gibson, 1993) than in separated capsules (maximum of 2–3 days).

About 10 days after oviposition, encapsulated larvae were placed in cultures containing potential metamorphic inducers. Cultures were maintained in tissue culture plates (Falcon Wells) containing 1 to 2 ml of fSW and a single inducer (defined below) at 17°C. About 15 to 20 larvae were placed in each well, depending on the size of the egg mass used and the number of wells required for the assay. At 24-h intervals, new hatchlings were counted and removed under a Wild M-5 microscope. Hatchlings were removed daily so that estimates would not include post-hatching metamorphosis (either spontaneous or in response to the tested inducer), which begins within 3 days of hatching (Gibson, 1993). Hatchlings were immediately scored as veligers or juveniles. Veligers were identified by the presence of an intact, ciliated velum and were either swimming or, less frequently, crawling. Individuals identified as metamorphosed juveniles had lost the velar cilia and the velar lobes had been resorbed (in many cephalaspideans, the shell is retained through the adult stage). Additional morphological changes (shell growth, elongation of the foot, acquisition of feeding structures and onset of feeding) occur within 2–3 days after hatching (Gibson and Chia, 1989a). The percentage of juveniles hatching in each well was determined for each day of the hatching period and is generally presented as a cumulative percent for each clutch by the end of the hatching period. Data are presented as the mean (± standard error) percentage of intracapsular metamorphosis occurring per treatment; data were arcsin transformed before further statistical analysis. Sample size refers to the number of egg masses (= number of clutches) used. Subgroups of larvae from each mass were simultaneously tested for each treatment per assay to allow an inter-clutch comparison of metamorphic rates. This comparison was necessary because larvae from different clutches are known to show variable rates of intracapsular metamorphosis (ranging from 4–100% in intact egg masses) arising from a genetically determined time of metamorphic competence (Gibson, 1993).

All assays contained two standard controls: (1) separated embryos cultured in fSW to determine the percentage of siblings per clutch undergoing spontaneous metamorphosis in absence of inducer; and (2) embryos cultured with untreated EMJ (about 1 mm^3 per well) to determine the proportion of embryos per mass competent to metamorphose during the assay period. Antibiotics were added to culture water to prevent degradation of EMJ or jelly extract (40 mg/l each of streptomycin sulfate and penicillin G).

Characterization and purification of the metamorphic inducer in egg mass jelly

EMJ was collected, drained of seawater, and stored frozen at either −9 or −60°C. About 1 mm^3 of untreated or experimentally treated EMJ (wet volume) was added to the indicated assay. In all cases, EMJ was thawed and thoroughly rinsed, first in distilled water and then in fSW, to remove any residual contaminants of each treatment.

The general stability of the metamorphic inducer in EMJ was determined by boiling (100°C, 20 min) or by lyophilizing (−46°C, 24 h) manually homogenized EMJ. Stability of inducer in the presence of acid was determined by acidifying an aqueous EMJ homogenate with acetic acid (0.1 *M*, 2 h). EMJ was also treated with one of two nonspecific proteases to determine if the inducer was proteinaceous. Proteinase K (final concentration 0.1 mg/ml) was added to 5 ml EMJ for 90 min at 18–20°C. Protease XIV from *Streptomyces griseus* (Sigma P-5147; final concentration 1 mg/ml) was added to 20 ml EMJ in 20 ml fSW at 17–20°C for 12 h, with continual mixing.

The molecular weight of the inducer was estimated with Amicon diaflo ultrafilters. We used a methanol (MeOH) EMJ extract, rather than EMJ pieces, in this bioassay to prevent clogging of the ultrafilters with the large mucoproteins composing much of the gelatinous matrix. Lyophilized EMJ (15 ml wet volume) was twice extracted (1 h each) with 25 ml MeOH. The combined extract was centrifuged (3000 rpm, 5 min.), the supernatant lyophilized, resuspended in distilled water, then filtered through both 5000 Da (Amicon YM5) and 1000 Da (Amicon YM2) ultrafilters (flow rates of 1 ml/2.5 min.). All solutions were lyophilized and resuspended in 30 ml fSW. Bioactivity was tested at several stages: (1) MeOH residue resuspended in distilled water before centrifugation; (2) distilled water suspension before filtration; (3) filtrates; and (4) residue from both YM5 and YM2 filters.

The inducer was isolated from EMJ by sequentially extracting the jelly with organic solvents of increasing polarity (20 ml each), including hexane, diethyl ether, 1:1 diethyl ether: methanol, methanol, and distilled water (Pawlik, 1986). Solvent was added to lyophilized EMJ (10 ml wet volume) and sonicated for 1 min, followed by extraction for 1 h with continual gentle agitation. Solvents were filtered, dried, resuspended in fSW, and added to each Falcon well (2-ml aliquots). Individual solvents were also tested (in absence of EMJ) for potential effects by evaporating an equivalent volume of solvent and resuspending any possible residue in fSW (*cf.* Pennington and Hadfield, 1989).

The metamorphic inducer was partitioned from the active fraction (MeOH extract) with HPLC (Spectra-Physics) using two buffer systems (gradient and isocratic). Bioactivity was determined for both crude MeOH extract (50 μl resuspended in 2 ml fSW) and HPLC fractions pooled over 2-min intervals (corresponding to 50 μl crude extract/2 ml fSW). Lyophilized EMJ was extracted with hexane to remove nonpolar compounds; eluant was discarded and EMJ dried. Residual EMJ was extracted with absolute MeOH as described above. The centrifuged MeOH extract was injected (100 μl) onto an Alltech RPC18 column (25 cm × 4.6 mm, 10 μm particle size) and partitioned on a buffer gradient from 100% methanol to 100% water over 30 min. Fractions were collected at 1-min intervals. All fractions were tested for inductive activity regardless of the presence of a visible peak (UV-vis detector set at 254 nm). Solvent was evaporated, the residue was resuspended in fSW, and the resuspension was added to Falcon wells and assayed as outlined above. Two fractions were found to contain activity (min 9–10, 17–18). To determine if both peaks represent the same or similar compounds, fractions containing the first peak of activity (min 9–10) were collected for three runs (300 μl original crude extract), concentrated in absolute MeOH, and repartitioned at the same gradient conditions. Fractions were collected and assayed as described above. In the final step of the purification procedure, the two active peaks (min 9–10, 17–18) were collected for three runs (300 μl crude extract), pooled, dissolved in MeOH, and subsequently reinjected through the same column under isocratic buffer conditions (absolute MeOH). Fractions were collected at intervals corresponding to visible peaks (at 254 nm) on the chromatograph, and both peaks and inter-peak intervals were assayed as outlined above.

Biological distribution of the metamorphic inducer

We examined specific tissues of adult *Haminaea callidegenita* for inductive activity. Tissues investigated were parapodial lobes, anterior and posterior mucous glands of the female reproductive system, gametolytic sac, and digestive gland. Tissue samples from three adults were combined, then extracted with MeOH and assayed as outlined above.

EMJ of four additional opisthobranch species was tested for ability to induce metamorphosis in *H. callidegenita* veligers. EMJ assayed for inductive potential came from the following opisthobranchs: *H. callidegenita; H. vesicula*, a sympatric congener that is morphologically and ecologically similar; *Melanochlamys diomedea*, a cephalaspidean found in the same habitat; *Alderia modesta*, an ascoglossan found in association with the zanthophyte *Vaucheria* sp. located in the high intertidal zone of the same bays as *H. callidegenita;* and *Onchidoris bilamellata*, a nudibranch found in rocky intertidal zones in association with barnacles. We tested both pieces of EMJ as well as a MeOH jelly extract for inductive potential on *H. callidegenita* larvae. Egg masses of the first three species were collected from False Bay and *Onchidoris* egg masses were collected at Friday Harbor Laboratories, both on San Juan Island, Washington State.

The metamorphic inducer found in *H. callidegenita* EMJ was tested on five other species of mollusc: the four opisthobranchs mentioned above (*H. vesicula, Melanochlamys diomedea, Alderia modesta, Onchidoris bilamellata*) and the oyster *Crassostrea gigas*. Planktotrophic veligers of all five species were cultured as by Kempf and

Willows (1977), except that antibiotics were not used and larvae were supplied with a 1:1:1 mixture of *Isochrysis galbana*, *Pavlova lutheri*, and *Rhodomonas* sp. as food. Larvae were identified as metamorphically competent when the propodium was well developed and the mantle was retracted from the shell (Bickell, 1978). These larvae were placed in 100-ml jars (n = 10 larvae/jar) containing fSW and one of the following substrates: EMJ (pieces of jelly or a MeOH extract); a metamorphosis-inducing substrate associated with that species; or 19 mmol K^+ in fSW. The latter two treatments were used to ensure that tested veligers were metamorphically competent. Metamorphosis-inducing substrates included diatoms and adult mucus for *H. vesicula* (Gibson and Chia, 1989b), sand containing nematodes for *Melanochlamys* (Gibson, pers. obs.), the alga *Vaucheria* for *Alderia* (Seeleman, 1933), the barnacle *Chthamalus dalli* for *Onchidoris* (Chia and Koss, 1988), and adult shell for *Crassostrea* (Crisp, 1967). All substrates were freshly collected from the adult habitat of each species. Competence was also determined using seawater containing excess potassium (19 mmol K^+), which is known to induce metamorphosis in several phyla of marine invertebrate (Yool *et al.*, 1986; Todd *et al.*, 1991). Percent metamorphosis was determined after 48 h.

EMJ from masses containing embryos of different developmental stages was assayed for inductive activity to test for effects of egg mass age. EMJ was separated and pooled from five egg masses at each of the following stages: gastrula; early veliger (showing cephalopedal rudiment); mid-veliger (statocysts visible, growth of larval shell complete); late veliger (eyes and heart well developed); and posthatch (all hatching was completed). EMJ was assayed as described above. Data were analyzed with a one-way ANOVA using the Scheffé procedure for unplanned comparisons among treatments (Day and Quinn, 1989).

Results

Characterization and isolation of the metamorphic inducer from egg mass jelly

Haminaea callidegenita larvae were induced to metamorphose within the embryonic capsule by egg mass jelly. Embryos cultured in the presence of EMJ metamorphosed within the embryonic capsule (X ± SE 52.68 ± 4.49% juveniles were released at hatching, n = 24 cultures) at rates expected of intact egg masses (61.32 ± 1.41% juveniles, n = 288 egg masses). Most embryos cultured in the absence of EMJ hatched as swimming veligers (2.12 ± 1.22% juveniles, n = 24 cultures of 15–20 larvae each). In assays involving pieces of EMJ, jelly was in the culture water but not necessarily in contact with developing embryos, indicating that the inducer is a water-soluble compound.

Inducer activity of EMJ was not destroyed by boiling (49% intracapsular metamorphosis was induced; Table I), lyophilizing (56%), or acidifying (46%) the jelly. Rates of intracapsular metamorphosis in these treatments were similar to rates observed in larvae cultured with untreated EMJ in the same experiment (67% intracapsular metamorphosis occurred; Table I). The metamorphic inducer passed through ultrafilters of both 5000 (46% metamorphosis) and 1000 molecular weight cutoff (36%; Table II), indicating that the inducer is less than 1000 Da in molecular weight. Activity was somewhat lower in treatments containing ultrafiltrate than in those with untreated EMJ (71%), but activity appeared to have been lost during extraction before filtration took place (the resuspended extract induced only 31% metamorphosis; Table II). Inductive potential was also unaffected by the action of two general proteases. EMJ treated with proteinase K induced 45% intracapsular metamorphosis, and 43% occurred in response to untreated EMJ (in absence of proteinase; Table III). Results were similar when EMJ was treated with pronase XIV, with 53% metamorphosis occurring in response to pronase-treated EMJ and 54% occurring in response to untreated EMJ (Table III).

The only organic solvent to extract significant amounts of inducer from EMJ was methanol (46% intracapsular metamorphosis; Table IV). Some inducer was retained by the EMJ, as evidenced by subsequent extraction of inducer with distilled water and the small amount of activity remaining in the EMJ after extraction (Table IV). The MeOH extract showed peak absorbance at 254 and a smaller peak at 303 nm, as well as a peak corresponding to the absorbance maximum for pure MeOH at 205 nm. Metamorphosis was not induced by potential residues left after evaporation of any of the organic solvents used in these extractions (Table IV). Spontaneous metamorphosis (in seawater only, and in absence of known cue) occurred in 2% of veligers tested, whereas 68% intracapsular meta-

Table I

Characteristics of the metamorphic inducer found in Haminaea callidegenita *egg mass jelly*

Treatment	n	% Metamorphosis
Seawater	11	5.36 ± 6.50
EMJ	11	67.36 ± 6.39
EMJ (100°C)	9	49.11 ± 8.29
EMJ (Lyophilized)	11	56.55 ± 4.36
EMJ (+ 0.1M H^+)	8	46.75 ± 8.74

Data are the mean (± standard error) percentage of intracapsular metamorphosis that occurred in response to EMJ or EMJ treated as indicated. Data from two controls are also listed, including rates of metamorphosis occurring in seawater (spontaneous metamorphosis) and rates of metamorphosis in response to untreated EMJ. n = number of egg masses tested.

Table II

Molecular weight estimates for the metamorphic inducer found in Haminaea callidegenita *egg mass jelly*

Treatment	*n*	% Metamorphosis
Seawater	6	6.95 ± 3.27
EMJ	6	71.34 ± 1.85
Resuspended extract	6	31.02 ± 5.87
<5000 Da Filtrate	6	46.53 ± 7.97
<1000 Da Filtrate	6	36.45 ± 6.12

Data are the mean (± standard error) percentage of intracapsular metamorphosis that occurred in response to EMJ extract and extract filtrates, as indicated. Data from two controls are also listed and include rates of metamorphosis occurring in seawater and in response to untreated EMJ. *n* = number of egg masses tested.

morphosis occurred in response to untreated EMJ (Table IV).

The inducer was partitioned from an EMJ extract in methanol with HPLC over a gradient from absolute MeOH to absolute water. The inducer eluted at 9–10 min (at a buffer concentration of about 70% MeOH) and at 17–18 min (about 40% MeOH) after injection (Fig. 1A), with some spreading of activity around the first active peak. Fraction 9–10 induced slightly less intracapsular metamorphosis (60%) than did EMJ (70%) or the crude MeOH extract (93%). Fraction 17–18 induced metamorphosis at a much lower rate (32%; Fig. 1A). Rates of intracapsular metamorphosis in cultures containing most other HPLC fractions were similar to rates in embryos cultured in seawater only. Fraction 9–10 was collected and pooled over three runs, then re-eluted under the same conditions to determine if the inducer would separate into two fractions. Again, the inducer eluted at two intervals, the first appearing 7–8 min after injection (59.19 ± 3.04% intracapsular metamorphosis) and the second at 15–18 min (27.48 ± 4.23%). This suggests that the inducer either racemizes under polar conditions or degrades during the elution procedure. Degradation of the inducer is less likely because the crude extract retains high levels of activity for at least several weeks (9°C).

The combined active fractions (min 9–10, 17–18) were re-eluted under isocratic buffer conditions (absolute MeOH). This resulted in a single peak of inducer eluting 8 min after injection (peak E in Fig. 1B), as well as the partitioning of several contaminants. Peak E was accumulated over several runs and the solvent was evaporated. A very small amount of dry residue remained as a fine, white powder that reacted strongly with vanillin, indicating the presence of steroids, phenols, or fatty acids as functional groups; alternatively, the strength of the vanillin reaction may indicate the presence of a residual contaminant, such as a polar lipid that may be derived from the presence of eggs in the original egg mass extract. The inducer residue also had a slight reaction with ninhydrin, indicative of the presence of primary amines. To detect possible contaminants, the pure fraction was re-eluted with HPLC and each fraction scanned with a refractive-index detector to reveal the presence of all compounds, regardless of specific ultraviolet or visual absorbance. The result was a single sharp peak, suggesting that this fraction represents pure inducer, although the presence of a contaminant eluting precisely with the inducer can not be eliminated.

Biological distribution

A metamorphic inducer was present in methanol extracts of several adult tissues, with most activity occurring in extracts of the posterior mucous gland of the female reproductive tract (61% intracapsular metamorphosis occurred), digestive gland (49%), and parapodial lobes (56%; Fig. 2). All three extracts induced intracapsular metamorphosis at rates comparable to that induced by untreated EMJ (48%). Less activity was evident in extracts of the anterior mucous gland of the female reproductive tract. Extracts of the gametolytic sac showed no activity (Fig. 2).

EMJ produced by other opisthobranch species also induced high rates of intracapsular metamorphosis in

Table III

The effects of two general proteases on the metamorphic inducer found in Haminaea callidegenita *egg mass jelly*

Proteinase K			Pronase XIV		
Treatment	*n*	% Metamorphosis	Treatment	*n*	% Metamorphosis
Seawater	8	7.6 ± 1.65	Seawater	12	6.79 ± 2.93
Untreated EMJ	8	43.75 ± 3.78	Untreated EMJ	12	54.56 ± 5.13
Proteinase treated EMJ	8	45.13 ± 8.19	Pronase treated EMJ	12	53.47 ± 6.21

Data are the mean (± standard error) percentage of intracapsular metamorphosis that occurred in response to EMJ or EMJ incubated with protease. Data from two controls are also listed, including rates of metamorphosis occurring in seawater (spontaneous metamorphosis) and rates of metamorphosis in response to untreated EMJ. *n* = number of egg masses tested.

Table IV

Extraction of the metamorphic inducer found in Haminaea callidegenita *egg mass jelly using sequential solvents of increasing polarity*

A. EXTRACTS			B. SOLVENT ONLY		
Treatment	*n*	% Metamorphosis	Treatment	*n*	% Metamorphosis
Seawater	12	2.27 ± 3.4	—	—	—
EMJ	10	68.00 ± 4.74	—	—	—
EMJ Extracts:					
Hexane	9	7.07 ± 2.71	Hexane	4	0.88 ± 0.62
Ether	6	1.43 ± 1.12	Ether	4	2.90 ± 1.05
Ether:MeOH 1:1	6	3.47 ± 1.76	Ether: MeOH 1:1	4	3.24 ± 1.22
MeOH	6	46.00 ± 5.62	MeOH	4	3.93 ± 1.42
Water	6	12.70 ± 3.88	Water	4	1.93 ± 1.69
Extracted EMJ	6	16.6 ± 6.12	—	—	—

Data are the mean (± standard error) percentage of intracapsular metamorphosis that occurred in response to EMJ or EMJ extract (column A), or residue of evaporated solvent (column B). Data from two controls are also listed: rates of metamorphosis occurring in seawater (spontaneous metamorphosis), and rates of metamorphosis in response to untreated EMJ. *n* = number of egg masses tested.

Haminaea callidegenita veligers (solid bars; Fig. 3). A similar response was observed in *H. callidegenita* veligers treated with a methanol extract of EMJ produced by these species (hatched bars; Fig. 3), suggesting that the inducer present in the jelly of other species is chemically similar to that produced by *H. callidegenita* (*i.e.*, also a small, polar compound). However, EMJ did not induce metamorphosis in any of the other four opisthobranch species tested (*H. vesicula*, *Melanochlamys*, *Alderia*, and *Onchidoris*; Fig. 4). Larvae of these four species were competent to metamorphose, as indicated by their response to excess K^+ in seawater as well as to species-specific metamorphic inducers (Fig. 4; substrates described in the Methods). Although veligers of the oyster *Crassostrea gigas* did metamorphose in response to EMJ pieces, they did not respond to a MeOH extract of EMJ (Fig. 4). Oyster larvae are known to metamorphose in response to microbial film (Coon *et al.*, 1985); thus these larvae were probably responding to bacteria associated with the jelly rather than to the specific inducer known to affect *H. callidegenita* veligers.

There were no significant differences in inductive activity of EMJ of different ages (Fig. 5), indicating that activity of the metamorphic inducer found in EMJ was not influenced by the age of the jelly (one-way ANOVA, $p \gg 0.05$).

Discussion

Opisthobranchs are often considered to have highly specific metamorphic cues that are derived from specific prey species or characteristics of post-metamorphic habitats. This generalization originates from widespread laboratory observations of metamorphosis but is based on only a few orders of opisthobranchs (especially nudibranchs, but also some anaspideans and ascoglossans), and exceptions have been noted within each order (in nudibranchs—Hubbard, 1988; in anaspideans—Pawlik, 1989). Metamorphic cues for larvae belonging to other opisthobranch orders are not as well understood and in cephalaspideans are known for three species only: *Haminoea solitaria* (Harrigan and Alkon, 1978) and *H. vesicula* (Gibson and Chia, 1989b), both known to settle on microbial film; and *H. callidegenita*, which has a highly specific inducer before hatching (EMJ). After hatching, *H. callidegenita* gradually becomes sensitive to a variety of additional substrates associated with small juveniles in the field and probably indicative of a juvenile food source (including the seagrass *Zostera marina*, the filamentous green alga *Chaetomorpha linum*, and sea lettuce, *Ulva* sp.). *H. callidegenita* veligers will also metamorphose in response to EMJ after hatching as they become competent (Gibson, 1993); therefore, this inducer acts both intracapsularly and on dispersive larvae.

Within the egg mass, approximately 60% of the *H. callidegenita* embryos respond to the EMJ inducer and hatch as juveniles. The remainder are released as swimming veligers and do not become competent to metamorphose until after hatching (range is 1–14 days posthatching; Gibson, 1993), although all veligers from a clutch eventually metamorphose successfully. The ecological result of this hatching pattern is poecilogonous development (variable development within one species) whereby every parent produces two types of offspring: swimming, lecithotrophic veligers with a higher potential for dispersal, and crawling juveniles that may recruit directly into the parental population. The percentage of metamorphosis that occurs within the egg mass is genetically determined, and 100% intracapsular metamorphosis occurs in rare cases only (<3% in more than 800 egg masses examined; Gibson, 1993).

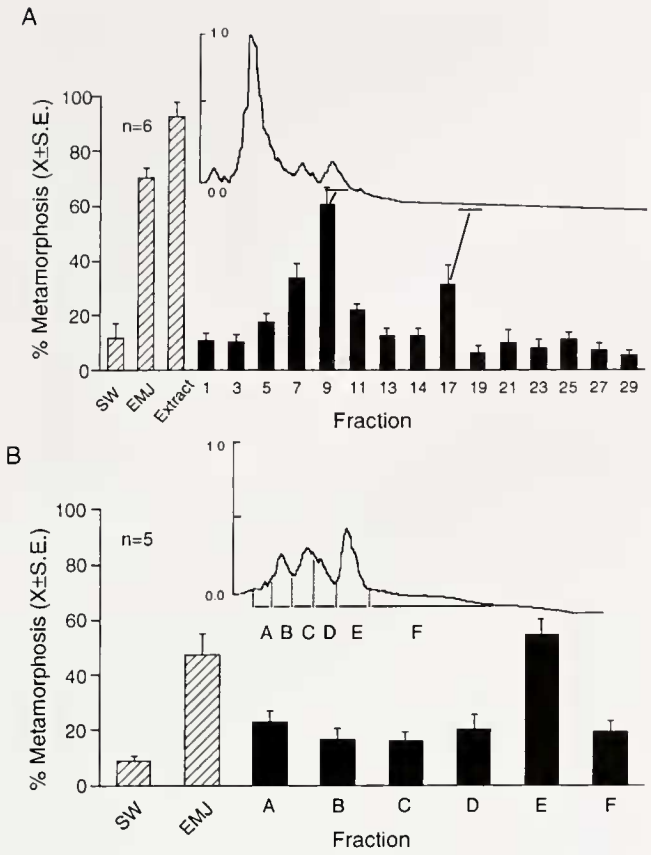

Figure 1. Isolation of metamorphic inducer with high performance liquid chromatography (HPLC). Data are the results of partitioning the inducer from a crude methanol extract with a buffer gradient from absolute methanol to absolute water (A) and partitioning the partially purified active fractions from A with an isocratic buffer system (absolute methanol) (B). In both A and B, the chromatograph indicates absorbance of each fraction as eluted from an RP-C18 column, detected at 254 nm, and the histogram gives results of the bioassay used to test the inductive potential of each fraction. In A, bioassay results are the percentage (mean ± standard error) of *Haminaea callidegenita* veligers that underwent intracapsular metamorphosis in response to seawater (SW), with untreated inducer (EMJ), with a crude EMJ extract (all in hatched bars), as well as with the indicated HPLC fractions (solid bars) pooled over 2-min intervals throughout a 30-min elution period. Peaks of activity are cross-referenced to the chromatograph. In B, results include the percentage of intracapsular metamorphosis occurring in response to standard controls (hatched bars) as well as in response to the fractions indicated in the chromatograph (solid bars). n = number of egg masses assayed.

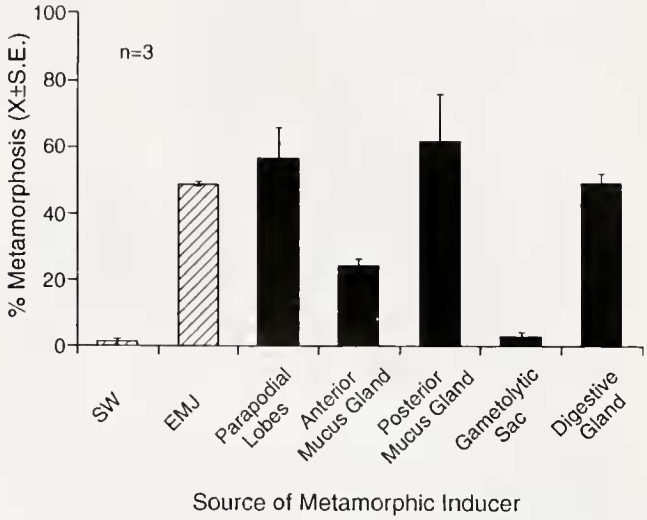

Figure 2. Activity of adult tissue as a metamorphic inducer. Data are the percentage of *Haminaea callidegenita* veligers that underwent intracapsular metamorphosis (mean ± standard error) in response to seawater (SW), untreated egg mass jelly (EMJ; both in hatched bars) as well as to a methanol extract of the indicated adult tissues (solid bars). n = number of egg masses assayed.

Isolation of the metamorphic inducer has been extensively studied in two species of opisthobranch, but in both cases the inducer has yet to be identified. Veligers of the nudibranch *Phestilla sibogae* are induced to metamorphose by a water-borne compound that is released from its prey, the hard coral *Porites* (Hadfield, 1977). This compound is small (200–500 Da) and stable to temperature and pH (Hadfield and Scheuer, 1985). The nudibranch *Eubranchus doriae* also metamorphoses in response to its prey species, the hydroid *Kirchenpaueria pinnata* (Bahamondes-Rojas, 1988). In this case, the inducer is water soluble and contains galactosidic residues. Metamorphosis of *E. doriae* is also induced by *cis*-isomers of various sugars (Bahamondes-Rojas and Dherbomez, 1990). The EMJ inducer that was active on *Haminaea*

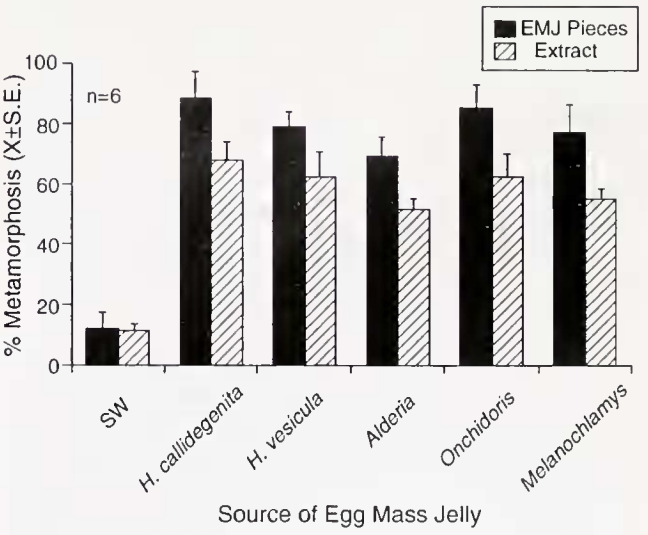

Figure 3. Metamorphic inducing activity of egg mass jelly (EMJ) produced by other opisthobranch species. Data are the percentage (mean ± standard error) of *Haminaea callidegenita* veligers undergoing intracapsular metamorphosis in response to seawater (SW), to EMJ pieces (solid bars), or to a methanol EMJ extract (hatched bars). Source of EMJ is indicated on the horizontal axis. n = number of *H. callidegenita* egg masses assayed.

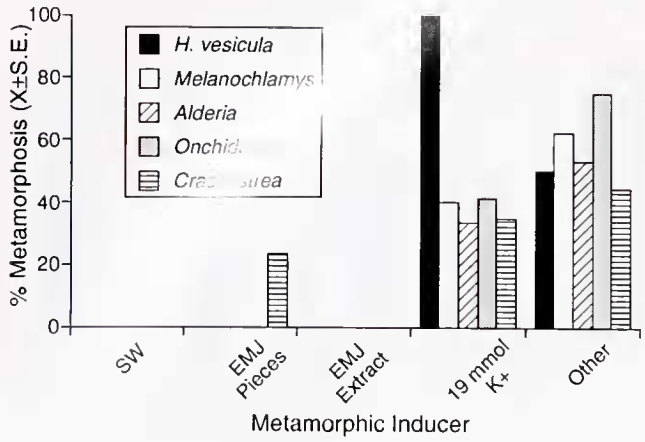

Figure 4. Specificity of egg mass jelly (EMJ) activity to *Haminaea callidegenita*. Data are the percentage of metamorphosis in veligers of five mollusc species (identified in the legend) in response to seawater (SW), EMJ, a methanol jelly extract, 19 mmol K^+, and a natural substrate associated with metamorphosis in that species (identified in text).

callidegenita veligers was a polar (methanol- and water-soluble) compound that was smaller than 1000 Da in molecular weight, non-proteinaceous, and stable to temperature (−60° to 100°C) and acid. It is interesting that veligers of all three opisthobranchs metamorphose in response to inducers that are small, non-proteinaceous, water-soluble compounds; however, further comparisons are not possible until these inducers have been identified. Under field conditions, metamorphic cues that are water soluble may become highly dilute within a short distance from the inducer source (Hadfield and Scheuer, 1985). Dilution of the EMJ inducer would presumably not be a problem as the compound need only diffuse through the egg mass to be effective (less than a few millimeters). Active inducer is present in jelly throughout the 2-week encapsulated period (the period from oviposition to hatching), suggesting that it is not released as the gelatinous matrix degrades during hatching. It is possible that the inducer is bound to a nonsoluble component of the egg mass (such as the matrix) or if it diffuses from the egg mass, it is present in large enough quantities to allow expected rates of metamorphosis.

The metamorphic inducer found in egg mass jelly appears to be derived from the parent, because methanol extracts of adult tissue contain a functionally similar compound. This potentially similar compound was isolated from most adult tissues assayed, suggesting that it is not uniquely produced or sequestered in the mucous glands of the reproductive tract (the organs that produce the egg mass; Rudman, 1971) but rather is found throughout most of the adult. Larvae of many benthic marine invertebrates are induced to metamorphose by conspecifics, including barnacles (*e.g.*, Knight-Jones, 1953), bivalves (*e.g.*, Veitch and Hidu, 1971), polychaetes (*e.g.*, Knight-Jones, 1951; Jensen and Morse, 1984), echinoids (Highsmith, 1982; Burke, 1984), and others (see reviews by Burke, 1986; Pawlik, 1992). This process causes gregarious settlement of larvae, either in response to adults (*e.g.*, Knight-Jones, 1953; Burke, 1984) or newly metamorphosed juveniles (*e.g.*, Cole and Knight-Jones, 1949; Leitz and Lange, 1991). Many advantages have been associated with this pattern of settlement, including identification of a habitat that will support juvenile and adult survival (Jensen, 1989) and the proximity of potential mates (Crisp, 1979; Pennington, 1985). Intracapsular metamorphosis in *H. callidegenita* is obviously not gregarious settlement (offspring hatching as juveniles have not settled), but advantages associated with a conspecific cue remain the same, both at the level of individual larvae (EMJ would identify a suitable juvenile habitat as one containing a reproductively active population of *H. callidegenita*) and the population (induction of intracapsular metamorphosis by EMJ would ensure continuous recruitment to the parental population). EMJ induces metamorphosis in veligers throughout the metamorphic period (Gibson, 1993); therefore, it may have a role in inducing gregarious settlement of hatched veligers, including those from other populations. However, its functional importance may primarily involve intracapsular metamorphosis, because a hatched veliger is less likely to encounter EMJ than the other, more widespread substrates known to induce metamorphosis posthatching (*e.g.*, *Zostera marina*).

A functionally similar metamorphic inducer is also found in the egg masses of other opisthobranch species,

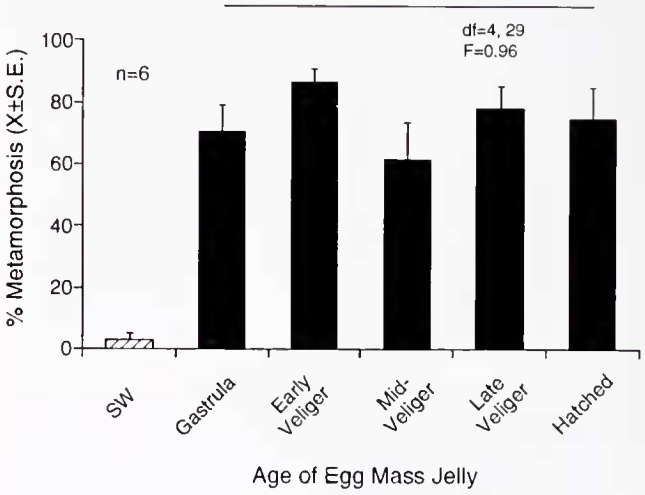

Figure 5. Effect of egg mass age on activity of the metamorphic inducer in egg mass jelly. Data are the percentage (mean ± standard error) of *Haminaea callidegenita* veligers that underwent intracapsular metamorphosis in response to seawater (SW) and egg mass jelly separated from egg masses at the stage indicated. The horizontal bar indicates that no significant differences were found among these groups (1-way ANOVA). n = number of egg masses assayed.

suggesting that this compound or group of similar compounds is intrinsic to the structure of these gelatinous egg masses. As such, production of the inducer, unlike the release of a more specific pheromone, would not require an additional expenditure of energy by the adult. Response of larvae to the inducer may be a consequence of opisthobranch veligers being sensitive to cues denoting a juvenile or adult food source. After hatching, juvenile *H. callidegenita* often graze diatoms and detritus from the surface of the egg mass and appear to ingest EMJ as well, before moving to nearby *Zostera* or *Chaetomorpha* (Gibson, pers. obs.)

Despite the fact that the inducing compound is not uniquely derived in *H. callidegenita*, its effects as a metamorphic inducer are restricted to this species. Competent veligers of five other molluscan species did not metamorphose in response to the metamorphic inducer in EMJ in a partially purified condition. This is not surprising for stenophagous species (in this study, *Onchidoris* and *Alderia* have restricted trophic requirements) as these larvae would be expected to metamorphose only on a highly specific substrate (Seeleman, 1933; Chia and Koss, 1988). *H. vesicula* coexists with *H. callidegenita* and adults of both species appear to have similar trophic requirements, but their larvae metamorphose in response to cues associated with more general characteristics of the habitat. Lack of response by these veligers to EMJ may reflect a higher probability of these long-lived veligers (4 weeks in *H. vesicula* and 6 weeks in *Melanochlamys;* Gibson, pers. obs.) contacting a more widespread cue than the less likely encounter with an egg mass after hatching. It would be interesting to test the EMJ inducer on opisthobranchs known to prey on the egg masses of other species, such as *Olea hanseensis* (Strathmann, 1987), which is also seasonally found in Padilla Bay (Gibson, pers. obs.).

The primary ecological effect of the presence of a metamorphic inducer in the egg mass is poecilogonous development: that is, by promoting intracapsular metamorphosis, this inducer allows for the release of both swimming veligers and crawl-away juveniles from each egg mass. Therefore, each parent produces dispersive propagules while simultaneously providing immediate recruits to the parental population. The second major factor shaping this developmental mode is variable intra-clutch time of competence, spanning both the hatching and posthatching larval periods (Gibson, 1993). The role of EMJ as an inductive substrate probably arose through a combination of two conditions: the nonspecific distribution of the chemical inducer (the inducer or a similar compound is found in adult tissue as well as in the egg masses of other opisthobranchs), and its role as a juvenile food source (possibly as a substrate from which to graze diatoms and detritus). To our knowledge, this is the first described example of larvae metamorphosing in response to a maternally derived egg mass, as well as the first description of an opisthobranch metamorphosing in response to conspecifics.

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